The Lichenologist **41**(6): 631–648 (2009) © British Lichen Society, 2009
doi:10.1017/S0024282909990120

Morphological and nuclear ribosomal DNA data support distinguishing two new species of *Umbilicaria* (*Umbilicariaceae*, Ascomycota) from Europe

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Abstract: *Umbilicaria iberica* and *U. maculata* are described as new to science on the basis of morphological and molecular data. *Umbilicaria iberica* is similar to *U. polyphylla* but differs in having a monophyllous thallus with a distinctive white reticular pattern over the umbilicus and actinodisc apothecia. *Umbilicaria maculata* is similar to *U. cylindrica* but this new species is distinguished by its grey-brown thallus with sparse marginal cilia and white stains on the upper surface especially in the marginal zone, as well as by sessile apothecia with one or a few sterile fissures. Nuclear ITS and LSU rDNA have been used as molecular markers. In the phylogenetic analysis *U. polyphylla* falls into two well supported clades (A and B), one of which corresponds to the morphotype that is described here as a new taxon. Specimens previously recognized as *U. cylindrica* fall into three well supported clades: clade C corresponds to the typical morphotype, clade D corresponds to morphotype II described here as a new taxon, and clade E is morphotype III which is recognized as *U.* cf. *umbilicarioides*. Typical group I introns have been found in position 1506 of the nuclear SSU rDNA of *Umbilicaria*; a consensus secondary structure of these introns is presented and we conclude that they represent an important and valuable molecular marker which gives additional support to the ITS and LSU sequence phylogeny obtained.

Key words: new species, *Umbilicaria iberica*, *Umbilicaria maculata*, ITS and LSU, introns

Introduction

Umbilicaria Hoffm. is a well-known genus of umbilicate, foliose, lichen-forming fungi comprising *c.* 90 currently accepted species with a worldwide distribution (Feuerer 2008), which occur almost exclusively on siliceous rocks in the polar, alpine and high-alpine biomes. One of the most important morphological and anatomical characters used to separate species is apothecium morphology (Frey 1933; Scholander 1934; Llano 1950). However, many individuals of *Umbilicaria* grow exclusively as anamorphs, and the important taxonomic characters for species identification are found on the asexual propagules, such as thalloconidia (Hasenhüttl & Poelt 1978; Hestmark 1990) or isidia and soredia (Krog & Swinscow 1986; Codogno *et al.* 1989; Wei *et al.* 1995–96; Sancho *et al.* 1998). Other important characters are the type of rhizinomorphs and subsequent structure of the upper and lower cortices of the thallus (Poelt 1977). Valladares & Sancho (1995) considered the structure of the medulla, such as differences in the degree of cohesion of hyphae and their spatial orientation within the medulla, as additional useful taxonomic characters for these lichens. However, taxonomic characters used for other lichen genera, such as secondary metabolic products, are of limited use for the identification of *Umbilicaria* species. According to Narui *et al.* (1996), most of the chemical

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https://doi.org/10.1017/S0024282909990120 Published online by Cambridge University Press

variation in *Umbilicaria* is due to the presence of small quantities of additional satellite compounds biosynthetically related to gyrophoric acid, such as umbilicaric, ovoic and hiascic acids. Most *Umbilicaria* species accumulate high concentrations of the tridepside, gyrophoric acid, always accompanied by smaller amounts of its depside precursor lecanoric acid. However, in the case of *U. cylindrica* (L.) Delise ex Duby the quantities of gyrophoric and lecanoric acids can differ between specimens, as well as the quality of other secondary products detected mainly by HPLC method (Narui *et al.* 1996; Seriña *et al.* 1996; Kantvilas & Louwhoff 2007).

In Europe, 31 species of *Umbilicaria* have been recognized (Feuerer 2008); 26 species occurring in the continental part of the Mediterranean region (Llimona & Hladun 2001; Nimis & Martellos 2003), 21 in the Carpathians (Bielczyk *et al.* 2004), and 25 in Fennoscandia (Santesson *et al.* 2004). The most variable species in the genus is *U. cylindrica,* and up to 6 varieties have been described from the Iberian Peninsula (Llimona & Hladun 2001), 5 from the Tatra Mountains in Poland (Krzewicka 2004), and 2 from Fennoscandia (Santesson *et al.* 2004). The characters used to distinguish these varieties are the frequency of rhizines and cilia, the concentration of wrinkles and folds on the upper surface, and the colour of the underside, as well as the occurrence of mono- or polyphyllous thalli. On the other hand, all the varieties of *U. cylindrica* have the same type of stipitate and gyrodisc apothecia and thalli with ciliate margins. Hitherto, in Europe only *U. cylindrica* has been characterized by the ciliate margin of the thallus and this feature has been used in many keys for European lichens as a good character for this species. For this reason, specimens occurring in the Tatra Mountains with a sparsely ciliate thallus and atypical sessile apothecia were previously considered by Krzewicka (2004) as *U. cylindrica*.

Specimens lacking rhizines and with multicellular thalloconidia, among other characteristic features, have been traditionally included under the name *U. polyphylla* (L.) Baumg. However, some specimens have some morphological modifications similar to typical specimens of *U. polyphylla*; for example, in the Iberian Peninsula one of us (LGS) has recorded two morphological forms of this species, the first with a monophyllous, dark brown thallus and white central part on the upper side, occurring mainly in mountainous regions, and the second with a polyphyllous, medium brown and uniformly coloured thallus, growing at both higher and lower altitudes. In the Carpathians, one of us (BK) has observed, together with polyphyllous uniformly brown coloured specimens, other morphotypes with a greyish brown, polyphyllous thallus, slightly areolate around the umbilicus. In all these cases, however, other characters mentioned above and typical of *U. polyphylla* are maintained.

Molecular data provide additional characters to test the validity of species circumscriptions. Nuclear ITS and large subunit ribosomal genes (LSU) have been used to examine relationships between closely related species of lichen-forming fungi, including *Umbilicaria* (e.g. Romeike *et al.* 2002; Ott *et al.* 2004; Divakar *et al.* 2005; Arup 2006; Søchting & Figueras 2007). Furthermore, the molecular phylogenetic relationships of the Lecanoromycetes (which includes the family *Umbilicariaceae*) were discussed by Wedin *et al.* (2005), Miądlikowska *et al.* (2006) and Hofstetter *et al.* (2007).

The present study aims to improve understanding of the variation in *U. cylindrica* and *U. polyphylla* using a combination of morphological data and DNA sequence data. Group I introns have been frequently reported in lichen-forming ascomycetes at a number of insertion sites in both the small (SSU) and large (LSU) subunits of the nuclear ribosomal genes (Martín *et al.* 2003). The presence and location of group I introns found in the SSU in *Umbilicaria*, representing their consensus secondary structure, are also reported.

Materials and Methods

Materials

Type and other lichen material were examined from KRAM–L and MAF herbaria. Ascomata and thallus

https://doi.org/10.1017/S0024282909990120 Published online by Cambridge University Press

material for nuclear ITS rDNA and nuclear LSU rDNA sequence analyses were obtained from 79 specimens representing 14 species of *Umbilicaria* and one species of *Lasallia*, used as an outgroup. Specimens for DNA analysis were air-dried at room temperature. Details of the materials and GenBank accession numbers are presented in Table 1.

The choice of *Lasallia pustulata* (L.) Mérat as an outgroup was based on the study by Ivanova *et al.* (1999). *Lasallia* is a closely related sister group to the paraphyletic genus *Umbilicaria* and both genera are placed in the *Umbilicariaceae* (Miądlikowska *et al.* 2006; Hofstetter *et al.* 2007).

Morphological and anatomical studies

Hand-cut sections of moist thalli were mounted in water containing a small amount of detergent and spot test reactions made with 10% KOH (K), a solution of sodium hypochlorite (C), and an alcoholic solution of paraphenylenediamine (Pd). Spore measurements and observations on ascomata structure were made on sections mounted in water or in *c.* 5% KOH. Chemical examination included response to ultraviolet light (UV) and thin-layer chromatography (TLC) which was performed in solvent system A and C (after Orange *et al.* 2001).

DNA extraction, PCR amplification

Total DNA was extracted from fresh, frozen and herbarium material. Small samples prepared from herbarium specimens were ground with sterile pestles in sterile Eppendorf tubes and extracted in acetone for 1 h to remove secondary lichen products. The acetone was discarded and the samples were dried at room temperature to allow the remaining acetone to evaporate. Total genomic DNA was extracted using E.Z.N.A. Fungal DNA Kit (OmegaBiotech) following the manufacturer's instructions.

Dilutions of the total DNA were used for PCR amplification of the nuclear ITS and LSU rDNA regions. Primers for amplification were (a) for the nuclear ITS rDNA: nu–SSU–1752–5′ (ITS1F) (Gardes & Bruns 1993) and nu–LSU–0041–3′(ITS4) (White *et al.* 1990), and (b) for the nuclear LSU rDNA: nuLSU–0042–5′ (LROR), nuLSU–0638–5′ (LR3), nuLSU–00952–3′ (LR5), LR7 – nuLSU–1422–3′ (LR7) (Vilgalys & Hester 1990). Individual reactions to a final volume of 25 µl were carried out using Ready-To-Go®PCR Beads (Amershan-Phamacia Biotech) with a 10 pmol μl^{-1} primer concentration (Martín & Winka 2000). The reactions were run with the following parameters for the nuclear ITS rDNA: initial denaturation at 95°C for 5 min, then 5 cycles of denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min, followed by 33 cycles of denaturation at 95°C for 30 sec, annealing at 48°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min and a 4°C soak; for the nuclear LSU rDNA: initial denaturation at 94°C for 5 min, then 36 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 1 min and 30 sec, with a final extension at 72°C for 10 min, and a 4°C soak. The PCR products were subsequently purified using the QIAquick Gel PCR Purification (Qiagen) according to the manufacturer's instructions. The purified PCR products were sequenced using the same amplification primers.

Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, Michigan, USA) was used to obtain the consensus sequence from the two strands of the ITS and/or the LSU nrDNA of each isolate.

Alignments and phylogenetic analyses

The ITS and LSU nrDNA sequences obtained were aligned separately using SeqApp (Gilbert 1993) for multiple sequences. Sequences obtained were compared with homologous sequences of *Umbilicaria* spp. retrieved from the EMBL Nucleotide Sequence Database included in Table 1. Where ambiguities in the alignment occurred, the one generating the fewest potentially informative characters was chosen. Alignment gaps were marked "–", unresolved nucleotides and unknown sequences were indicated with "N". Introns were not included in these analyses, but were studied separately to examine their distribution, secondary structure and phylogenetic origin.

From each data set, a maximum parsimony analysis (MP) was carried out; minimum length Fitch trees were constructed using heuristic searches with tree-bisection-reconnection (TBR) branch swapping, collapsing branches if maximum length was zero and with the MulTrees option on in PAUP*4.0b10 (Swofford 2003). Gaps were treated as missing data. Nonparametric bootstrap support (Felsenstein 1985) for each clade, based on 10 000 replicates using the fast-step option, was tested. The consistency index, CI (Kluge & Farris 1969), retention index, RI (Farris 1989), and rescaled consistency index, RC (Farris 1989) were obtained.

A combined matrix of ITS and LSU nrDNA sequences was analysed by parsimony using PAUP* and by a Bayesian approach (Larget & Simon 1999; Huelsenbeck *et al.* 2001) using MrBayes 3.1 (Ronquist & Huelsenbeck 2003). The combined matrix included sequences of those specimens from which both regions were obtained. As a measure of congruence between the ITS and the LSU data sets, the incongruence length difference (ILD) test (Farris *et al.* 1995) was performed in PAUP* with 10 000 replicates of the partition homogeneity test using the default search parameters except the MulTrees option off, keeping the best tree per analysis.

The model used for the Bayesian analyses was determined according to the Akaike Information Criterion (AIC) as implemented in Modeltest 3.7 (Posada & Crandall 1998). For the combined ITS-LSU matrix, a General Time Reversible model with a gamma shaped distribution of rates across sites and a proportion of in invariable sites (GTR+I+Γ) was selected. Two independent and simultaneous analyses starting from different random trees were run for 2 000 000 generations with four parallel chains and trees and model scores saved every 100th generation. The default priors in MrBayes

https://doi.org/10.1017/S0024282909990120 Published online by Cambridge University Press

TABLE 1. *Specimens used in the study, with location, reference collection detail and GenBank accession numbers. Sequences obtained from GenBank are in bold*

Species	Locality and collector(s)	Herbarium	GenBank acc. no.	
			ITS	LSU
U. antarctica 1	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2001	AY603123	AY603107
U. antarctica 2	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2002	AY603124	AY603108
U. antarctica 3	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2003	AY603125	AY603109
U. antarctica 4	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2005	AY603127	AY603111
U. antarctica 5	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2006	AY603128	AY603112
U. antarctica 6	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2004	AY603126	AY603110
U. antarctica 7	Antarctica, King George I., *J. Smykla* 63a/02	KRAM-L-47126	**FN185922**	–
U. antarctica 8	Antarctica, King George I., *J. Smykla* 58a/02	KRAM-L-47128	**FN185923**	**FN186056**
U. antarctica 9	Antarctica, King George I., *J. Smykla* 62a/02	KRAM-L-47130	**FN185924**	–
U. antarctica 10	Antarctica, King George I., *J. Smykla* 62b/02	KRAM-L-47130	–	**FN186057**
U. antarctica 11	Antarctica, King George I., *J. Smykla* 03a/96	KRAM-L-47127	**FN185925**	**FN186058**
U. antarctica 12	Antarctica, King George I., *J. Smykla* 03b/96	KRAM-L-47127	**FN185926**	**FN186059**
U. antarctica 13	Antarctica, King George I., *J. Smykla* 61a/02	KRAM-L-47122	**FN185927**	**FN186060**
U. antarctica 14	Antarctica, King George I., *J. Smykla* 61b/02	KRAM-L-47122	**FN185928**	**FN186061**
U. antarctica 15	Antarctica, King George I., *J. Smykla* 61c/02	KRAM-L-47125	**FN185929**	**FN186062**
U. antarctica 16	Antarctica, King George I., *J. Smykla* 57a/02	KRAM-L-47137	**FN185932**	**FN186064**
U. aprina 1	Iran, *M. Sohrabi*, 111660a	KRAM-L-53304	**FN185930**	**FN186063**
U. aprina 2	Iran, *M. Sohrabi*, 111660b	KRAM-L-53304	**FN185931**	–
U. crustulosa	Norway, *Lumbsch* 12165a	F	–	AY300869
U. cylindrica 1	Ukraine, collection number 48, unpublished	–	AF096209	–
U. cylindrica 2	Poland, Tatra Mts, *B. Krzewicka* 3173a	KRAM-L-53232	**FN185933**	**FN186065**
U. cylindrica 3	Poland, Tatra Mts, *B. Krzewicka* 3173b	KRAM-L-53232	**FN185934**	**FN186066**
U. cylindrica 4	Poland, Tatra Mts, *B. Krzewicka* 921	KRAM-L-46783	**FN185935**	**FN186067**
U. cylindrica 5	Poland, Tatra Mts, *B. Krzewicka* 3172a	KRAM-L-53233	**FN185936**	**FN186068**
U. cylindrica 6	Poland, Tatra Mts, *B. Krzewicka* 3172b	KRAM-L-53233	**FN185937**	**FN186069**
U. cylindrica 7	Poland, Tatra Mts, *B. Krzewicka* 871b	KRAM-L-45538	**FN185938**	**FN186070**
U. cylindrica 8	Poland, Tatra Mts, *B. Krzewicka* 3174a	KRAM-L-53234	**FN185939**	**FN186071**
U. cylindrica 9	Poland, Tatra Mts, *B. Krzewicka* 3174b	KRAM-L-53234	**FN185940**	**FN186072**
U. cylindrica 10	Poland, Tatra Mts, *B. Krzewicka* 3171	KRAM-L-53235	**FN185982**	**FN186101**
U. cylindrica 11	Poland, Tatra Mts, *B. Krzewicka* 566b	KRAM-L-53305	**FN185942**	**FN186074**
U. decussata	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2007	AY603122	AY603113

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TABLE 1. *Continued*

Species	Locality and collector(s)	Herbarium	GenBank acc. no.	
			ITS	LSU
U. hirsuta	Spain, El Escorial, *B. Krzewicka* 3299	KRAM-L-53226	–	**FN186075**
U. hyperborea	Norway, *Wiklund* 25	UPS	–	AY853399
U. iberica 1	Spain, El Escorial, *B. Krzewicka* 3291	KRAM-L-50626	**FN185964**	**FN186076**
U. iberica 2	Spain, El Escorial, *B. Krzewicka* 3292	KRAM-L-50627	**FN185965**	**FN186077**
U. iberica 3	Spain, El Escorial, *B. Krzewicka* 3290	KRAM-L-50625	**FN185966**	–
U. kappenii 1	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2008	AY603129	AY603114
U. kappenii 2	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2009	AY603130	AY603115
U. kappenii 3	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2010	AY603131	AY603116
U. kappenii 4	Antarctica, Livingston I., *H.T. Lumbsch* 19047a & *L. Sancho*	F	AY603132	AY603117
U. krascheninnikovii	Antarctica, Livingston I., *H.T. Lumbsch* 19046b & *L. Sancho*	F	AY603134	AY603118
U. maculata 1	Poland, Tatra Mts, *B. Krzewicka* 920a	KRAM-L-53236	**FN185967**	**FN186078**
U. maculata 2	Poland, Tatra Mts, *B. Krzewicka* 871a	KRAM-L-53237	**FN185968**	**FN186079**
U. maculata 3	Poland, Tatra Mts, *B. Krzewicka* 3042	KRAM-L-53240	**FN185969**	**FN186080**
U. maculata 4	Poland, Tatra Mts, *B. Krzewicka* 3040	KRAM-L-53238	**FN185970**	**FN186081**
U. maculata 5	Poland, Tatra Mts, *B. Krzewicka* 3041a	KRAM-L-53239	**FN185971**	**FN186082**
U. maculata 6	Poland, Tatra Mts, *B. Krzewicka* 3041b	KRAM-L-53239	**FN185972**	**FN186083**
U. maculata 7	Poland, Tatra Mts, *B. Krzewicka* 566a	KRAM-L-53241	**FN185973**	**FN186084**
U. nylanderiana 1	Antarctica, Livingston I., *H.T. Lumbsch* 19046b & *L. Sancho*	F	AY603133	AY603119
U. nylanderiana 2	no data, unpublished		AF096205	–
U. nylanderiana 3	Poland, Tatra Mts, *B. Krzewicka* 3045	KRAM-L-50628	**FN185974**	**FN186085**
U. polyphylla 1	Norway, *Wiklund* 32	UPS	–	AY853400
U. polyphylla 2	Poland, Tatra Mts, *B. Krzewicka* 825a	KRAM-L-45553	–	**FN186086**
U. polyphylla 3	Poland, Tatra Mts, *B. Krzewicka* 825b	KRAM-L-45553	–	**FN186087**
U. polyphylla 4	Poland, Tatra Mts, *B. Krzewicka* 659a	KRAM-L-45517	–	**FN186088**
U. polyphylla 5	Poland, Tatra Mts, *B. Krzewicka* 659b	KRAM-L-45517	–	**FN186089**
U. polyphylla 6	Poland, Tatra Mts, *B. Krzewicka* 3039a	KRAM-L-53225	–	**FN186090**
U. polyphylla 7	Poland, Tatra Mts, *B. Krzewicka* 3039b	KRAM-L-53225	–	**FN186091**
U. polyphylla 8	England, Cumbria, *B. Krzewicka* 3067a	KRAM-L-50619	–	**FN186092**

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TABLE 1. *Continued*

Species	Locality and collector(s)	Herbarium	GenBank acc. no.	
			ITS	LSU
U. polyphylla 9	England, Cumbria, *B. Krzewicka* 3067b	KRAM-L-50619	–	**FN186093**
U. polyphylla 10	Slovakia, Tatra Mts, *B. Krzewicka* 3044a	KRAM-L-53224	–	**FN186094**
U. polyphylla 11	Slovakia, Tatra Mts, *B. Krzewicka* 3044b	KRAM-L-53224	–	**FN186095**
U. polyphylla 12	Poland, Tatra Mts, *B. Krzewicka* 3046	KRAM-L-50618	**FN185975**	**FN186096**
U. polyphylla 13	Spain, El Escorial, *B. Krzewicka* 3296	KRAM-L-50621	**FN185976**	**FN186097**
U. polyphylla 14	Spain, El Escorial, *B. Krzewicka* 3297	KRAM-L-50622	**FN185977**	**FN186098**
U. polyphylla 15	Spain, El Escorial, *B. Krzewicka* 3298	KRAM-L-50623	**FN185978**	**FN186099**
U. polyphylla 16	Spain, El Escorial, *B. Krzewicka* 3293	KRAM-L-50620	**FN185979**	–
U. umbilicarioides 1	Antarctica, Lagoon I., *S. Ott*	hb. Ott 2011	AY603121	AY603120
U. cf. *umbilicarioides 2*	Romania, Rodna Massif, *K. Wilk* 3552a	KRAM-L-53303	**FN185980**	**FN186100**
U. cf. *umbilicarioides 3*	Romania, Rodna Massif, *K. Wilk* 3552b	KRAM-L-53303	**FN185981**	–
U. cf. *umbilicarioides 4*	Poland, Tatra Mts, *B. Krzewicka* 3170	KRAM-L-53242	**FN185941**	**FN186073**
U. vellea 1	no data, unpublished	–	AF096208	–
U. vellea 2	no data, isolate sm11005, unpublished	–	AF297668	–
U. vellea 3	Poland, Tatra Mts, *B. Krzewicka* 3043a	KRAM-L-53231	**FN185983**	**FN186102**
U. vellea 4	Poland, Tatra Mts, *B. Krzewicka* 3043b	KRAM-L-53231	**FN185984**	**FN186103**
OUTGROUP				
Lasallia pustulata 1	Spain, Madrid, El Escorial, *B. Krzewicka* 3295	KRAM-L-53227	**FN185985**	–
Lasallia pustulata 2	Sweden, 5 June 2001 *M. Wedin*	UPS	–	AY300839

were used in the analysis. Every 1000th generation tree from the two runs was sampled to measure the similarities between them and to determine the level of convergence of the two runs. The potential scale reduction factor (PSRF) was used as a convergence diagnostic and the first 25% of the trees were discarded as burn-in before stationarity was reached. Both the 50% majority-rule consensus tree and the posterior probability of the nodes were calculated from the remaining trees with MrBayes. Phylogenetic trees were drawn using TreeView (Page 1996).

Finally, secondary structures of the group I introns were based upon sequence alignments taking into account detailed, but general, features (Michel & Westhof 1990; Martín *et al.* 2003; Wikmark *et al.* 2007). Secondary structure diagrams were drawn using the Adobe Illustrator package (CS3-Version 9). A maximum parsimony analysis (MP) was carried out from the introns with the same parameters used for the ITS nrDNA, LSU nrDNA and ITS-LSU nrDNA analyses.

Results

Morphology

Two morphotypes (I and II) in the *U. polyphylla* group were distinguished (Table 2). Morphotype I was characterized by its polyphyllous, rarely monophyllous thallus; upper surface uniformly coloured, pale to dark brown, glossy, smooth, epruinose;

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TABLE 2. *Principal differences between* Umbilicaria polyphylla *and* U. iberica *(clades A and B)*

Character	morphotype I (A) *U. polyphylla*	morphotype II (B) *U. iberica*
Thallus	polyphyllous	monophyllous
Upper surface	uniformly coloured, pale to dark brown, glossy, smooth, epruinose	grey-brown to dark brown, at centre whitish, dull, weakly wrinkled, pruinose
Apothecia	gyrodisc	actinodisc
Medulla	deusta type	havaasii type
Lower surface	completely black or with brown margin	completely black or with pruinose margin
Thalloconidia	6–10-cellular, dark brown, rugged, 16–26 × 15–20 μm	3–5(8)-cellular, dark brown, scabrous, (10–) 15·3 (–20) × (10–) 13·3 (–20) μm

lower surface sooty black, smooth, covered with a fine layer of black, multicellular thalloconidia, lacking rhizines; gyrodisc apothecia. This morphotype corresponds with typical specimens of *U. polyphylla*. Additional information on its morphological features can be found in Llano (1950), Hestmark (1990), Krzewicka (2004) and Kantvilas & Louwhoff (2007). Morphotype II differs from typical specimens of *U polyphylla* by its monophyllous thallus; upper surface grey-brown to dark brown, whitish at the centre, dull, slightly scabrous and pruinose, elevated and white areolate in the centre; lower surface sooty black, smooth, covered with a fine layer of black, multicellular thalloconidia, lacking rhizines; actinodisc apothecia. This morphotype corresponds with the new species described here as *U. iberica* (for a complete description see below).

Three morphotypes (I, II and III) were distinguished in the *U. cylindrica* group (Table 3). Morphotype I was characterized by its ciliate, mono- or polyphyllous thallus, upper surface pale to dark grey, lower surface pale brown to pinkish-beige, smooth, rhizines sparse to dense, concolorous with the lower cortex; apothecia gyrodisc, black, with smooth or slightly cracked margin, stipitate, 0·5–2·0(–4·0) mm diam., disc black, gyrose. This morphotype corresponds with typical *U. cylindrica*; for more detailed descriptions see Llano (1950) and Narui *et al.* (1996), Krzewicka (2004) and Kantvilas & Louwhoff (2007). Morphotype II differs from typical *U. cylindrica* by: a monophyllous thallus with sparse, flattened cilia, upper surface smooth, grey to brownish-grey with white irregular smudges sometimes appearing UV+ white (structural effect), lower surface smooth, pale cream to whitish, with sparse to dense rhizines concolorous with the lower cortex; apothecia omphalodisc, black, with crenate margin, sessile to slightly stipitate, up to 0·5–0·8(–1·2) mm diam., disc black, smooth with one large or a few smaller sterile fissures. This morphotype corresponds with the new species described here as *U. maculata* (see below for complete description). Morphotype III is characterized by: a polyphyllous or rarely monophyllous thallus with margin covered by dark brown to black rhizines, upper surface grey to brown-grey, lower surface scabrous, medium brown, rhizines in marginal zone, concolorous with the lower cortex or darker, multicellular thalloconidia on rhizines (not observed in material studied from Europe). This morphotype is recognized here as *U.* cf. *umbilicarioides* (Stein) Krog & Swinscow due to the small number of samples, lack of thalloconidia on European materials and disjunct distribution. For additional descriptions of *U. umbilicarioides* see Krog & Swinscow (1986), Hestmark (1990), Øvstedal & Lewis Smith (2001), Krzewicka & Smykla (2004) and Kantvilas & Louwhoff (2007).

Molecular analyses

Nuclear ITS rDNA

The new 44 fungal ITS nrDNA sequences were aligned with 18 sequences available in Genbank to produce a matrix of 895

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TABLE 3. *Principal differences between the morphotypes of the* Umbilicaria cylindrica *complex (clades C, D and E)*

Character	morphotype I (C) *U. cylindrica*	morphotype II (D) *U. maculata*	morphotype III (E) *U.* cf. *umbilicarioides*
Thallus	mono- or polyphyllous, ciliate	monophyllous, only young lobes with sparse cilia	polyphyllous, with marginal rhizines giving ciliate appearance
Apothecia	gyrodisc, stipitate, margin smooth	omphalodisc, sessile, margin crenate	gyrodisc, stipitate, margin slightly incised
Upper surface	grey, uniformly coloured, slightly wrinkled	grey to brown-grey, with white stains, smooth	grey to brown-grey, mottled, areolate-scabrid
Lower surface	pale brown to pinkish, smooth	pale cream to white, smooth	medium brown, scabrous
Rhizines	cylindrical, simple to weakly branched, without thallyles	flattened to cylindrical, moderately branched, without thallyles	cylindrical, moderately to richly branched, with thallyles
Thalloconidia	absent	absent	multicellular, on rhizines

unambiguously aligned nucleotide position characters. The first 337 characters were eliminated in the phylogenetic analyses since there is a group I intron inserted after position 1506 in the SSU rRNA gene (*Escherichia coli* numbering system). These intron sequences are analysed separately (see below). From the 558 characters, 388 were constant, 64 variable and parsimony uninformative, and 106 parsimony informative. Maximum parsimony (MP) analysis under heuristic search gave 100 most parsimonious trees with a length of 326 steps, CI= 0·6442, RI= 0·8759 and RC= 0·5643. The alignment of 62 sequences and the strict consensus tree obtained from the MP analysis are available in TreeBASE (http://www.treebase.org/).

Nuclear LSU rDNA

The new 49 fungal LSU nrDNA sequences were aligned with 18 sequences available in Genbank to produce a matrix of 950 unambiguously aligned nucleotide position characters, of which 769 were constant, 97 variable were parsimony uninformative, and 84 parsimony informative. Maximum parsimony (MP) analysis under heuristic search gave 100 most parsimonious trees with a length of 281 steps, CI = 0·7153, RI = 0·9018 and RC = 0·6451. The alignment of 67 sequences and the MP strict consensus tree are available in TreeBASE.

Combined ITS and LSU nrDNA

The partition homogeneity test (ILD test) revealed significant congruence between the ITS and LSU data partitions (P = 0·7905) and therefore both data sets were combined for further analyses. The combined matrix had 1533 unambiguously aligned nucleotide position characters, of which 1203 were constant, 158 variable were parsimony uninformative, and 172 parsimony informative. The alignment of 51 sequences is available in TreeBASE. Maximum parsimony (MP) analysis under heuristic search gave 100 most parsimonious trees with a length of 529 steps, CI = 0·7183, RI = 0·8736 and RC = 0·6276. The trees obtained from the MP and Bayesian analyses gave similar topologies; thus only the Bayesian tree from the combined analysis is shown in Fig. 1. Branches with posterior probabilities (pp) are indicated in bold. The bootstrap support values (bs) equal or above 85% are indicated on the branches.

In the majority-rule consensus tree of the combined data set (Fig. 1), clade I comprising the *Umbilicaria polyphylla* complex is strongly supported. Its sister group relationship to *U. nylanderiana* (Zahlbr.) H. Magn.,

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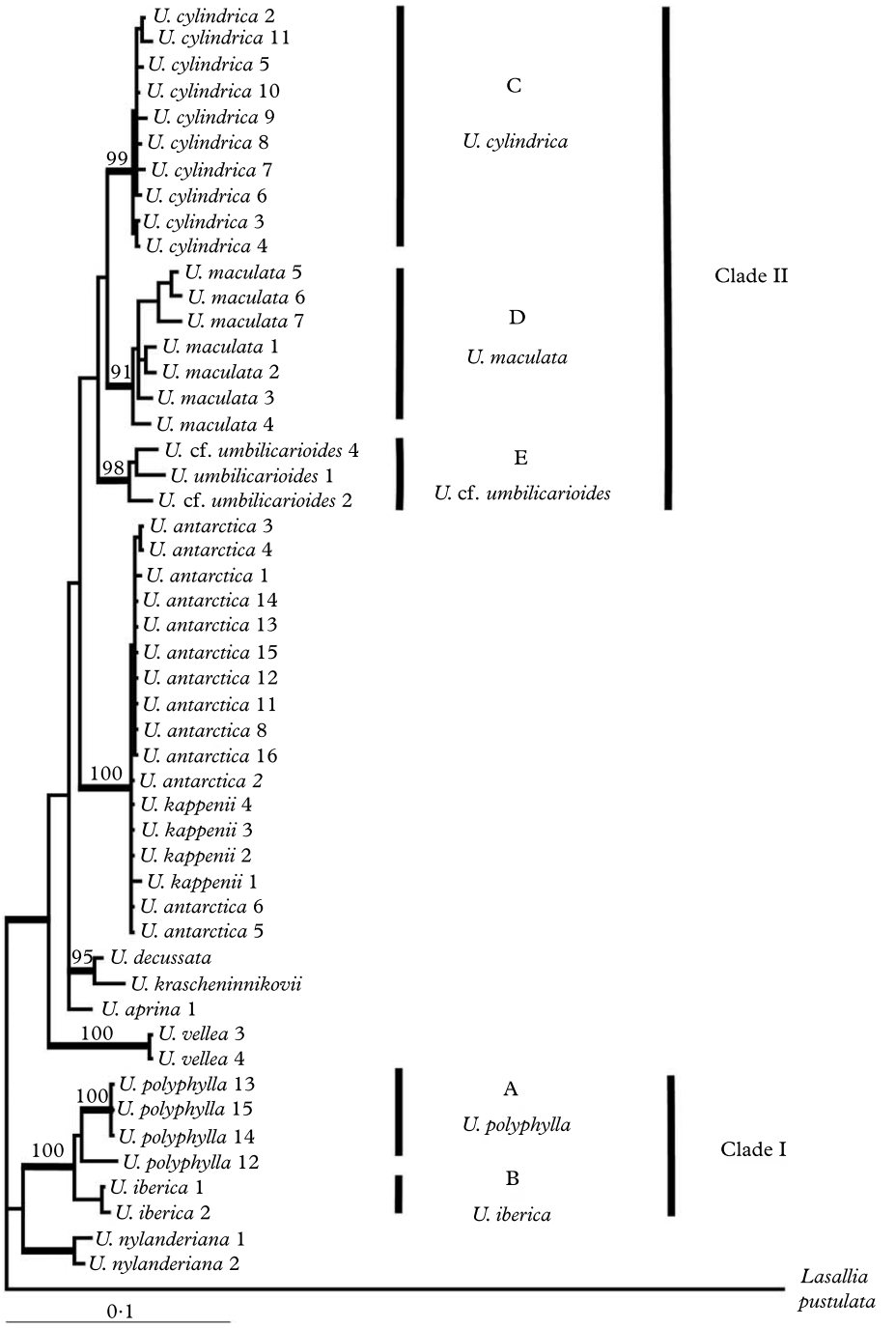

FIG. 1. Phylogeny of *Umbilicaria* spp. as inferred from a nuclear ITS and LSU rDNA combined data set. This is a 50% majority–rule consensus tree from a B/MCMC tree sampling procedure. Bold branches with posterior probabilities equal to or above 0·99. MP bootstrap support values above 85% are indicated on the branches.

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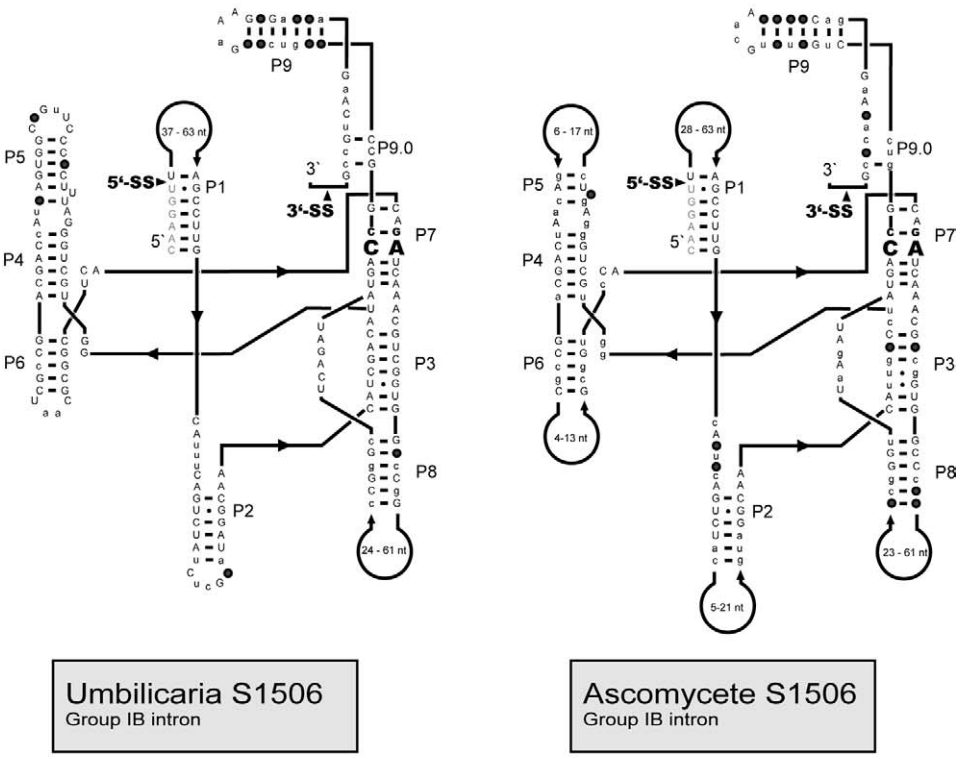
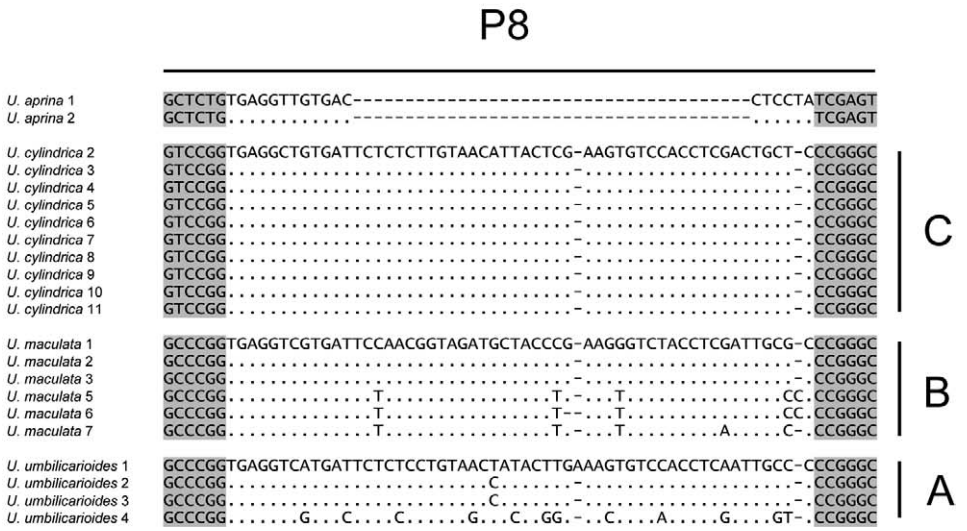
A



B



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however, is not well supported (pp = 0·56, bp = 50). All the remaining taxa are included in a group with low bp support (60). In the upper part of the tree, the supported group includes the two collections of *U. vellea* (L.) Hoffm. examined in this study; this group appears as the sister-group to the remaining taxa, however, without support. These taxa are clustered in three clades: one comprising the sequences of *U. antarctica* Frey & I.M Lamb and *U. kappenii* Sancho, Schroeter & Valladares obtained from GenBank, and the new sequences of *U. aprina* Nyl.; a second clade, which is very well supported, has sequences of *U. decussata* (Vill.) Zahlbr. and *U. krascheninnikovii* (Savicz) Zahlbr. from GenBank; and a third clade comprising sequences obtained in this study under the *U. cylindrica* complex and the *U. umbilicarioides* sequence from GenBank (clade II). Only clades I and II will be discussed.

Clade I contains clades A (pp = 0·63, bs = 61) and B (pp = 1·00, bs = 100) (Fig. 1). In the analyses of the nrLSU rDNA sequences (not shown), the collection *U. polyphylla* 12 forms a well–supported clade together with 10 new sequences and one sequence from GenBank (AY853400). Clade A contains specimens which are recognized here as morphotype I, representing the typical specimens of *U. polyphylla*. Clade B comprises sequences from two collections that correspond to morphotype II of the *U. polyphylla* group (Table 2). In our analyses, this morphotype is highly supported as monophyletic (pp = 1·00, bs = 100; Fig. 1) and is described here as *U. iberica* (Fig. 4).

In Clade II, three groups (C, D and E) are very well supported: Clade C (pp = 1·00, bs = 92) comprises 10 sequences from specimens that fit the morphology of the typical *U. cylindrica* (Morphotype I; Table 3); Clade D (pp = 1·00, bs = 92) contains seven sequences that correspond to morphotype II, here recognized as *U. maculata* (Fig. 5; Table 3); Clade E (pp = 1·00, bs = 87) includes two sequences previously identified as *U. cylindrica* and one from GenBank named as *U. umbilicarioides*. The analyses of the ITS nrDNA also included a GenBank sequence (AF096209) from a specimen collected in Ukraine (not shown). The morphology of the specimens of Clade E fit morphotype III (Table 3), recognized here as *U.* cf. *umbilicarioides*.

SSU rDNA intron

An insertion at the SSU rDNA position 1506 was found in *U. aprina* and *Umbilicaria* sequences included in clade II (except *U. cylindrica* 1 and *U. maculata* 4). The insertion represents a typical group IB intron. These introns are small (less than 300 bp), and a consensus secondary structure diagram is presented in Fig. 2A (left). From the 313 unambiguously aligned nucleotide position characters, 213 were constant, 13 variable were parsimony uninformative, and 86 parsimony informative. Maximum parsimony (MP) analysis under heuristic search gave 100 most parsimonious trees with a length of 133 steps, CI = 0·8647, RI = 0·9421 and RC = 0·8146. The alignment of 22 sequences and the MP strict consensus tree (Fig. 3) are available in TreeBASE.

Discussion

Based on morphological and molecular data, the morphotype II of *U. polyphylla* (Clade I, B) and morphotype II of *U. cylindrica* (Clade

FIG. 2. Analysis of *Umbilicaria* group IB intron secondary structure and variability. A, consensus secondary structure diagram based on 22 *Umbilicaria* introns sequences (left) and corresponding ascomycete introns (right). Paired elements (P1–P9) are indicated, and 100% invariable nucleotide positions are shown in uppercase letters. The atypical A : C pair in P7 is indicated in bold characters. For the *Umbilicaria* structure (left), variable positions present in 75% or more of the sequences are shown in lowercase letters, whereas those less than 75% conserved are indicated by solid circles. Similarly, positions common to two of the three genera (*Umbilicaria*, *Teloschistes*, and *Symbiotaphrina*) are shown in lowercase letters. Nucleotide positions present in all the genera, but different types, are shown as solid circles. B, sequence alignment of the P8 extension region. Nucleotides representing the six base pairs shown in Fig. 2A are marked as grey boxes. The A, B, and C clades are shown (right).

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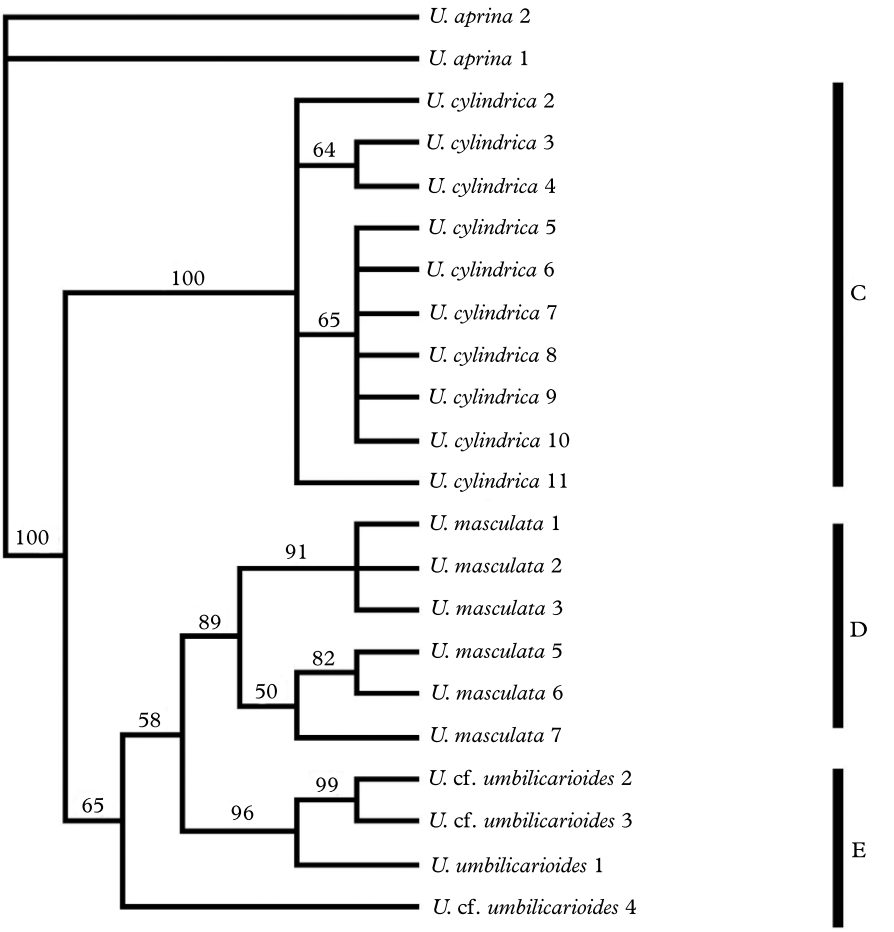

FIG. 3. Strict consensus tree of the maximum parsimony analysis of *Umbilicaria* group IB intron. MP bootstrap support values above 50% are indicated on the branches.

II, D) are described here as new species, *U. iberica* and *U. maculata* respectively.

Umbilicaria iberica is well supported as monophyletic, and is morphologically clearly distinguished from typical specimens of *U. polyphylla* (Table 2). The other collections in Clade I do not form a well-supported monophyletic group. It is interesting to note that Clade A (Fig. 1, Clade I) includes only sequences of collections of *U. polyphylla* from Spain, whereas the other sequences of specimens from temperate and boreal parts of Europe (e.g. Norway, Britain, Poland and Slovakia) form, together with *U. polyphylla* 12 and the GenBank (AY853400) sequence of *U. polyphylla* from Norway (not shown), a sister clade to the Spanish specimens.

In Clade II, three monophyletic groups are present among samples previously referred to as *U. cylindrica* (morphotype I – C, morphotype II – D, morphotype III – E). Our study agrees with previous studies in showing that *U. cylindrica* is probably a species complex including several cryptic species; for instance, chemical differences among specimens of this taxon were observed by Narui *et al.* (1996), morphological differences by Crespo & Sancho (1978), and Krzewicka (2004), and physiological differences by Fahselt *et al.* (1995). Interesting information

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is provided by the position of the sequences of *U. cylindrica* var. *corrugatoides* Frey (*U. cylindrica* 10) and var. *delisei* Nyl. (*U. cylindrica* 7) on the phylogenetic tree (Fig. 1). Although these varieties have a very different morphology, such as structure and colour of upper and lower sides of the thallus, their sequences are grouped in Clade C together with those of typical *U. cylindrica*. Morphologically, these three groups (C–E) are very similar, but all of them are highly variable. However, some of them are well-recognized by their sexual propagules, for example *U. maculata* (Table 3). Furthermore, molecular characters clearly distinguish these three groups (C–E) in the analyses of both ITS and LSU nrDNA sequence data and the presence of a group I intron in the position 1506 of the SSU nrDNA (Fig. 2).

The relationship of the specimens in Clade E is most unexpected since it comprises sequences of the morphotype III of *U. cylindrica* and the GenBank AY603120 sequence of *U. umbilicarioides* from Antarctica (Ott *et al.* 2004). To date, *U. umbilicarioides* was known only from the Southern Hemisphere, namely Antarctica (Øvstedal & Lewis Smith 2001), Africa (Krog & Swinscow 1986), South America (Llano 1950) and the Australian region where it was mostly known under the name *U. propagulifera* (Vain.) Llano (Galloway 1985). This species is easily distinguished by its multicellular thalloconidia produced by multidivided rhizines, which are missing on specimens of *U. cylindrica*. The specimens studied of morphotype III are characterized by upper and lower surfaces similar to *U. umbilicarioides* in colour and structure, and also in the distribution of rhizines. However, they lack the multicellular thalloconidia that are an important taxonomic character in many keys (e.g. Øvstedal & Lewis Smith 2001; Krzewicka & Smykla 2004). Phylogenetic analyses show that asexual propagules are not reliable taxonomic characters in *Umbilicaria* (Ott *et al.* 2004). The morphotypes, with and without thalloconidia, form one phylogenetic species that exhibits a high plasticity in developmental morphology and reproductive strategy. Furthermore, the specimens of morphotype III from Europe occasionally form thallyles on the top of rhizines, which are characteristic of *U. umbilicarioides* and have never been reported from *U. cylindrica*. The thallyles are situated on rhizines which differ from the regular rhizines in being unbranched, longer and thicker, being similar to those described on *U. umbilicarioides* by Krog & Swinscow (1986). On the basis of morphological and molecular data of nuclear ITS and LSU rDNA, the sequences in clade E probably belong to *U.* cf. *umbilicarioides*.

SSU rDNA intron

The introns found in *Umbilicaria* represent typical group IB intron folds, which are highly unusual in nuclear rDNA but present in some lichen-forming ascomycetes (see Martín *et al.* 2003). Comparison of group IB introns at SSU rDNA position 1506 from *Umbilicaria*, *Teloschistes*, and the non-lichen forming *Symbiotaphrina* identify a highly conserved catalytic core region within an almost identical intron fold (Fig. 2A, right). Group IB introns are generally valuable genetic markers among closely related isolates due to strong vertical inheritance patterns (Martín *et al.* 2003; Wikmark *et al.* 2007). The unusually small and compact core structure depends on host factors for self-splicing, and thus the group IB introns are more integrated into the host genetic systems than most other nuclear group I introns. This idea is further supported by an unconventional A : C basepair adjacent to the catalytical essential G : C basepair in the P7 segment (indicted by large bold characters in Fig. 2A), which probably affects catalysis.

Similar to the previously reported group IB introns in *Teloschistes*, the *Umbilicaria* cognate introns have size and sequence variations within the P8 segment. These sequences appear too short to be included in regular phylogenetic analysis, but may represent an informative synapomorphy among the *Umbilicaria* isolates. A closer inspection of the P8 region strongly supports this hypothesis (Fig. 2B). Whereas the two intron sequences from *U. aprina* possess a P8 of

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only *c.* 35 nucleotides, all the *U. cylindrica* isolates contain *c.* 70 nucleotides at this region. Furthermore, three sequence groups are recognized by simple alignments among *U. cylindrica* and correspond quite well to the C, D, and E clades (Fig. 3) obtained with the ribosomal RNA genes and ITS sequences. We conclude that the group IB introns represent an important and valuable molecular marker which gives additional support to the ITS and LSU sequence phylogeny presented above.

The Species

Umbilicaria iberica Sancho & Krzewicka sp. nov.

Thallus monophyllus, umbilicatus, orbiculatus, 1–3(–5) cm diametro, margine saepe incise–lobato. Superficies superior laevis vel tenuiter scabrida, fuliginosa, ad centrum pruinosa ac elevata. Superficies inferior laevis, omnino nigra vel fusca ad margine. Apothecia sessilia, actinodiscus. Ascosporae ellipsoideae, 11–13 × 6·0–7·5 µm. Thalloconidia 3–5(8)–cellularia, subglobosa vel ellipsoidea, (10–) 15·3 (–20) × (10–) 13·3 (–20) µm.

Typus: Spain, El Escorial near Madrid, on a hill above the town, on shaded rocks, 1070 m alt., 17 September 2006, *B. Krzewicka* 3292 (KRAM–L 50627—holotypus; MAF & hb. M.R.D. Seaward 115716—isotypi).

(Fig. 4)

Thallus monophyllous, up to 3(–5) cm diam., margins revolute, entire or somewhat lacerate. Upper surface dull, smooth to weakly wrinkled, grey-brown to dark brown, at the centre elevated, slightly radially wrinkled, white areolate-scabrid and pruinose. *Medulla* white, one-layered, plectenchyma scarcely branched, hyphae loose, with many open intercellular spaces, rarely occupied by gelatinous matter. Lower surface completely sooty black, or with paler marginal zone (often pruinose), or mottled, smooth. *Rhizines* absent.

Apothecia occasionally present, sparse, black, sessile to substipitate, up to 0·7–1·5 mm diam., actinodisc. *Epihymenium* dark brown, up to 15 µm thick. *Hymenium* hyaline, up to 75 µm thick. *Paraphyses* usually simple, non-septate, up to 2·5 µm thick. *Asci* clavate, 35–40 × 10–13 µm. *Ascospores* 8 per ascus, simple, hyaline, 11–13 × 6·0–7·5 µm.

Pycnidia occasional to frequent, dark brown to black, punctiform, 0·1–0·2 mm diam. *Conidia* bacillar 3·5–5·0 × 1·0 µm. *Thalloconidia* common, multicellular, 3–5(8)-cellular, spherical to ellipsoid, (10–) 15·3 (–20) × (10–) 13·3 (–20) µm, covering lower surface completely or in black patches.

Chemistry. TLC: gyrophoric and umbilicaric acids; medulla C+ red, K−, Pd−, KC−, thallus UV−.

Ecology and distribution. At present, this species is known only from Spain where it occurs on vertical surfaces of blocks of siliceous rocks in shaded places. *Umbilicaria iberica* has been observed by L. Sancho in many places in the Iberian Peninsula, and it probably also grows in Mediterranean and sub-Mediterranean regions of Europe and Africa.

Note. The new species is distinguished from *U. polyphylla* by its monophyllous thallus, elevated and white areolate part over the umbo, actinodisc apothecia and a different type of medulla (Table 2).

Selected material examined. **Spain:** Bajada del Pto. de la Morcuera Canchal, NE exposition, 1450 m, 10 x 2006, *L. G. Sancho* (hb. M.R.D. Seaward; KRAM; MAF); El Escorial near Madrid, on a hill above the town, on shaded rocks, 1070 m, 2006, *B. Krzewicka* 3290, 3291 (KRAM).

Umbilicaria maculata Krzewicka, M. P. Martín & M. A. García sp. nov.

Thallus parvus, monophyllus, maculiformis, umbilicatus, orbiculatus, 1–3 cm diametro. Superficies superior ad margines laevis, centrum versus vel areolato–scabrida. Superficies inferior laevis, albescens. Rhizinomorphae plerumque albescens, ramosae vel perrarae simplices, applanatae vel cylindricae irregulariter dispersae. Apothecia sessilia, omphalodiscus. Ascosporae latae ellipsoideae, 10–12 × 5–6 µm.

Typus: Poland, Western Carpathians, High Tatra Mts, Mały Kozi Wierch Mt., 49°13′12″N, 20°01′13″E, on granite rock, 2220 m alt., 10 September 2005, *B. Krzewicka* 3040 (KRAM-L 53238—holotypus; hb. M.R.D.Seaward 115717—isotypus).

(Fig. 5)

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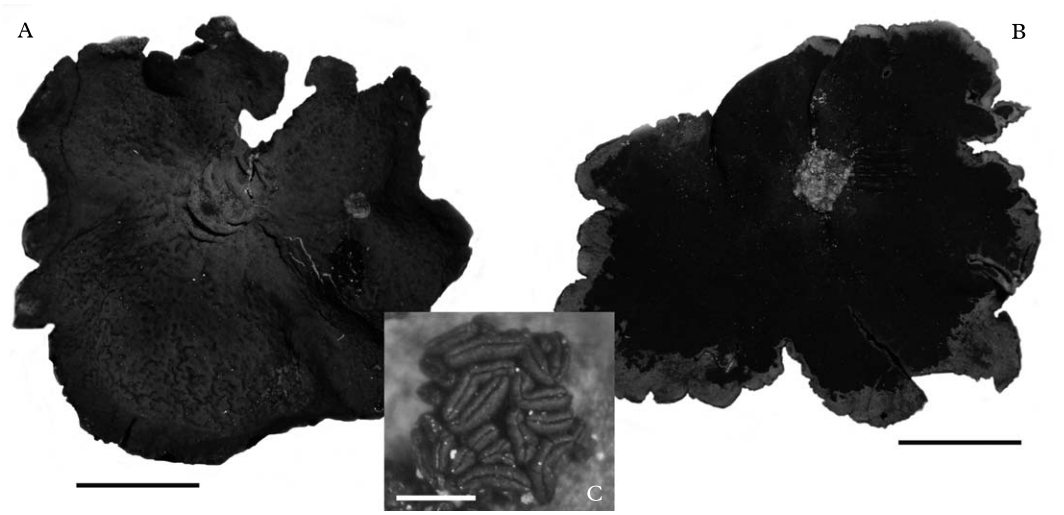

FIG. 4. *Umbilicaria iberica* (KRAM–L 50627). A, upper surface of thallus; B, lower surface of thallus; C, actinodisc apothecium. Scales: A & B = 5 mm; C = 0·5 mm.

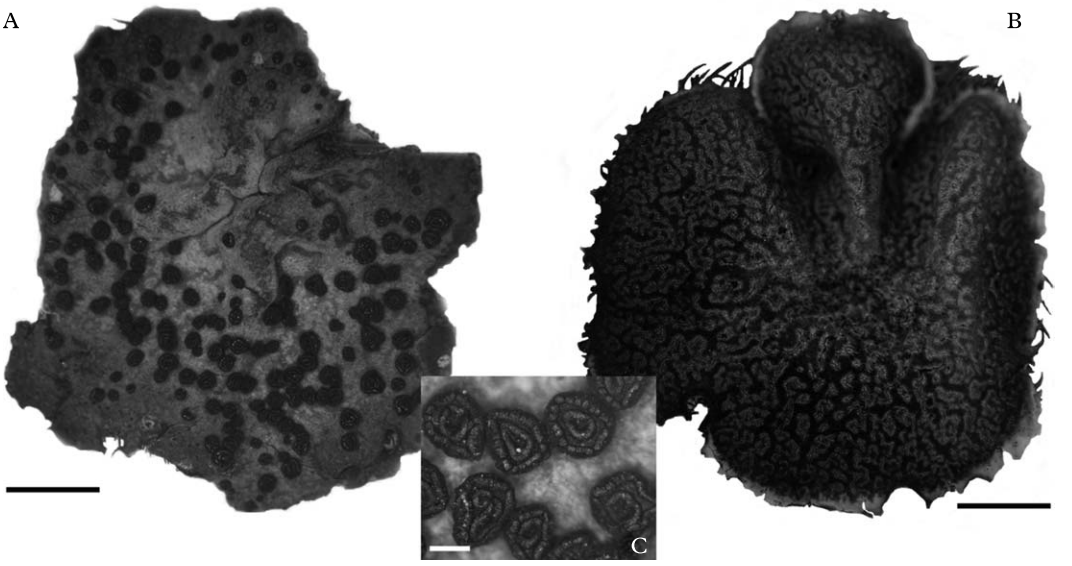

FIG. 5. *Umbilicaria maculata.* A, upper surface of thallus with apothecia (KRAM–L 53238); B, upper surface of sterile thallus (KRAM–L 53239); C, omphalodisc apothecia. Scales: A & B = 3 mm; C = 0·5 mm.

Thallus monophyllous, flattened, adhering to the substratum, up to 3 cm diam., with or without sparse marginal cilia on young lobes. Upper surface smooth, dull, grey to grey–brown, white in the places where the cortex is thinner giving a maculate appearance and UV+ (structural effect), in the centre white and scabrous to areolate. *Medulla* white, two–layered, the upper part loose, with an arachnoidal plectenchyma, the lower part

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with compact plectenchyma. *Lower surface* smooth, dull, pale creamy to white, darker towards the margin. *Rhizines* concolorous with the lower cortex, simple to branched, mainly dichotomously to moderately divided, flattened, rarely cylindrical, area around the umbilicus without rhizines, towards the margin rhizines more abundant, scattered.

Apothecia frequent, sparse, black, sessile to substipitate, up to 1(–2) mm diam., omphalodisc with large sterile fissure in the centre or with a few sterile fissures, disc margin with characteristic marginal incisions. *Epihymenium* dark brown, up to 20 µm thick. *Hymenium* hyaline, up to 50 µm thick. *Paraphyses* usually simple, non–septate, up to 2·5 µm thick. Asci clavate, 40–45 × 10–15 µm. *Ascospores* 8 per ascus, simple, hyaline, 10–12 × 5–6 µm.

Pycnidia common, moderately numerous, distributed in the marginal zone, immersed, onion–shaped, with brown wall and dark brown ostiole, conidiophores unbranched, septate. *Conidia* cylindrical, 4·0 × 0·8 µm. *Thalloconidia* absent.

Chemistry. TLC: no lichen substances detected; medulla C−, K−, Pd−, KC−, thallus UV−.

Ecology and distribution. This species occurs on vertical surfaces of large blocks of siliceous rocks in rather shaded and wind–exposed places. To date it has been reported only from the Tatra Mts in Poland, where several populations have been observed at higher altitudes by the first author. The species grows in alpine and subnival belts, but probably occupies a wider area where it may have been overlooked or have been misidentified as *U. cylindrica*.

Notes. *Umbilicaria maculata* is characterized by having a monophyllous, flattened, maculate thallus adhering to the substratum, with sparse or lacking cilia, and sessile omphalodisc apothecia with crenulated margin. *Umbilicaria cylindrica* can be distinguished from this species by its stipitate gyrodisc apothecia and the thallus more or less revolute-convolute with dense and long cilia. However, its juvenile forms of apothecia look like those of *U. maculata*.

Selected material examined. **Poland:** *Western Carpathians*: High Tatra Mts below Rysy summit, on granitic rock, 2225 m, 1999, *B. Krzewicka* 920a (KRAM); High Tatra Mts, Mięguszowiecka Przełęcz pod Chłopkiem pass, on granitic rock, 2307 m, 1999, *B. Krzewicka* 871a (KRAM); High Tatra Mts, Mały Kozi Wierch Mt, 2220 m, on granitic rock, 2005, *B. Krzewicka* 3042 (KRAM); High Tatra Mts, Kozia Przełęcz Pass, 2137 m, on granitic rock, 2005, *B. Krzewicka* 3041 (KRAM, MAF); High Tatra Mts, Hala Gąsienicowa alp. Żółta Turnia Mt, on W slope, 1860 m, on granitic block, 1999, *B. Krzewicka* 566a (KRAM).

We are much obliged to Prof. M. R. D. Seaward who generously read the manuscript, revised the English and made a number of valuable comments. We are grateful to Dr W. Paul (Institute of Botany PAS, Krakow) for correcting the Latin diagnosis. Financial support of the European Community's Programme "Structuring the European Research Area", under a SYNTHESYS grant to BK (ES–TAF 1431) at the Real Jardín Botánico (CSIC) and a Spanish grant, CGL2005–06549, are gratefully acknowledged.

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Accepted for publication 16 *May* 2009

https://doi.org/10.1017/S0024282909990120 Published online by Cambridge University Press